

**Genetic characterization of the marine ichthyotoxic
flagellate *Pseudochattonella farcimen* (Heterokonta)
and phylogenetic relationships among heterokonts.**

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of philosophiae doctor**

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Blindern Oslo, March 2008

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Abstract

In most cases, the proliferation of marine planktonic alga is ecologically beneficial for the marine food web. However, harmful algal blooms can have negative effects by causing fish kills. In this PhD thesis particularly one ichthyotoxic phytoflagellate that has caused massive blooms in Scandinavian waters since its first live record in 1998, has been studied.

The first objective of this thesis was to determine the phylogeny and systematic position of this organism. The species name *Pseudochattonella farcimen* sp. nov. was proposed as it was found to be different from, but closely related to *P. verruculosa*, previously described as the Raphidophyte *Chattonella verruculosa* Y. Hara et Chihara from Japan. Ultrastructure, morphology and pigment composition as well as phylogenetic analyses of nuclear rDNA confirmed that the genus *Pseudochattonella* belong to the heterokont class Dictyochophyceae – and not Raphidophyceae as previously believed. Florenciellales, a new order within the Dictyochophyceae was proposed, which embraces the three species *Florenciella parvula* Eikrem, *P. farcimen* and *P. verruculosa*.

A further aim of this thesis was to genetically characterize *P. farcimen* and related species, and determine the genetic diversity within and between *Pseudochattonella* species. Genetic evidence for a separation of the two *Pseudochattonella* species was found in nuclear rDNA as well as in protein coding DNA sequences from mitochondria and chloroplast. Another objective was to develop molecular methods for detection of this species, as well as determine the identity of bloom-forming *Pseudochattonella* species in various geographical regions.

Finally this work was brought into a broader perspective as *P. farcimen* was included in a multigene phylogenetic analysis. Bayesian and maximum likelihood analyses improved the heterokont tree compared to previous rDNA analyses. Except for the positioning of Chrysophyceae, Eustigmatophyceae and Pinguiphyceae, all main branches of Ochrophyta were resolved. Further all plastid-free heterotrophic heterokonts were placed sister to Ochrophyta with robust support.

List of papers

This thesis is based on the following four papers, which will be referred to in the text by their Roman numerals.

- I.** Edvardsen B., Eikrem W., Shalchian-Tabrizi K., Riisberg I., Johnsen G., Naustvoll L., Throndsen J.
Verrucophora farcimen gen. et sp. nov. (Dictyochophyceae, Heterokonta) a bloom forming ichthyotoxic flagellate from the Skagerrak, Norway.
Published. Journal of Phycology (2007) 43: 1054-1070.
- II.** Riisberg I., and Edvardsen B.
Genetic variation in bloom-forming ichthyotoxic *Pseudochattonella* species (Dictyochophyceae, Heterokonta) using nuclear, mitochondrial and plastid DNA sequence data.
Submitted to European Journal of Phycology (October 2007).
- III.** Riisberg I., and Edvardsen B.
Molecular probes and specific PCR primers for detection and identification of ichthyotoxic marine flagellates in the genus *Pseudochattonella* (Dictyochophyceae, Heterokonta).
Submitted to Journal of Plankton Research (February 2008).
- IV.** Riisberg I., Orr R. J. S., Kluge R., Shalchian-Tabrizi K., Bowers H. A., Patil V., Edvardsen B. and Jakobsen K.S.
Seven gene phylogeny of heterokonts.
To be submitted to Protist.

List of abbreviations and definitions

Axenic culture	in phycology, a laboratory-maintained single strain or algal species that is free of other algae, bacteria or fungi.
Bp	Base pair
Blast	Basic Local Alignment Search Tool.
Bigyra	a phylum within Heterokonta which, together with Pseudofungi constitute heterotrophic heterokonts.
Concerted evolution	(or horizontal evolution) mechanisms by which mutations in a repeat can spread “horizontally” to all members in the same gene family.
<i>cox1</i>	cytochrome oxidase subunit I.
EST	Expressed Sequence Tag.
GO	Gene Ontology (provides a controlled vocabulary to describe gene and gene product attributes in any organism).
Ichthyotoxic	toxic to fish, fish-killing.
Intraspecific variation	variation within a species.
Interspecific variation	variation among species.
ITS rDNA	internal transcribed spacer regions of ribosomal DNA.
Mucocysts	saclike structures within cells from which thick, rod-shaped mucilage can be extruded to the cell surface when the organism is disturbed.
Ochromphyta	a division within Heterokonta that includes all photoautotrophic members.
LSU rDNA	large subunit ribosomal DNA, or alternatively 28S rDNA.
Parenchyma	(adj. parenchymatous) a form of cell tissue.
<i>psbA</i>	photosystem II <i>psbA</i> protein.
<i>rbcL</i>	rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) large subunit.
<i>rbcS</i> – <i>rbcL</i> spacer	the rubisco spacer region lies downstream of the <i>rbcL</i> gene, between the <i>rbcL</i> and <i>rbcS</i> genes.
Rhizoplast	a striated, contractile strand that extends from the flagellar basal bodies into the cell, often connecting with the nuclear surface.
SSU rDNA	small subunit ribosomal DNA, or alternatively 18S rDNA.
Taxon	a general term for any taxonomic category.
Thallus (pl. thalli)	The body of an alga, which is not differentiated into vascularized leaves, roots, and stems.

Background

1.1 Eukaryotic marine harmful algal blooms and ichthyotoxic algae

Algae include some of the most abundant eukaryotes on earth, and together with land plants they are responsible for the bulk of global primary productivity. Algae (including cyanobacteria) are crucial for life on earth as they are the major source of food for marine life. Planktonic algae or phytoplankton, living in the oceans perform nearly half of the global photosynthesis (Behrenfeld and Falkowski, 1997). In most cases, the proliferation of planktonic algae is beneficial for aquaculture and fisheries. However, in some situations algal blooms (up to millions of cells per liter) can have a negative effect causing severe losses to aquaculture and fisheries. Harmful algal blooms can cause several problems for fish, as some algae produce toxins that are directly harmful and even fatal for fish (Brodiet and Lewis, 2007). Another widespread problem for fish farmers is the production of fatty acids or galactolipids which damage the epithelial tissue of the gills. Algae can also cause problems by physical clogging of gills, by mucus excretion, or production of oxygen radicals (Brodiet and Lewis, 2007). Virtually all algal-blooms, even non-toxic species, reduce the fishes' appetite and reduce oxygen concentrations, stress the fish and make them vulnerable to diseases. Several algal species in European marine waters, mainly within the divisions Dinophyta, Haptophyta and Heterokonta cause ichthyotoxic (fish-killing) harmful algal blooms (HAB).

In this PhD thesis I have studied heterokont algae, and the focus in the following will therefore be limited to heterokonts. Within heterokonts several species in the classes Raphidophyceae (e.g. *Chattonella marina* (Subrahman) Hara and Chihara, *Chattonella antiqua* (Hada) Ono, *Fibrocapsa japonica* Toriumi and Takano, *Heterosigma akashiwo* (Hada) Hada ex Y. Hara et Chihara) and Bacillariophyceae (*Pseudo-nitzschia*) cause HAB. Fish kills have also been associated with blooms of *Dictyocha speculum* Ehrenberg (Dictyochophyceae) (Henriksen, 1993).

1.2 The infrakingdom Heterokonta

Resolving the phylogenetic relationships between eukaryotes is an ongoing challenge of evolutionary biology (Burki et al., 2007). A current hypothesis for the tree of eukaryotes proposes that all diversity can be classified into five or six putative very large assemblages, the so-called ‘supergroups’. These comprise the ‘Opisthokonta’ and ‘Amoebozoa’ (often united in the ‘Unikonts’), ‘Archaeplastida’ or ‘Plantae’, ‘Excavata’, Chromalveolata, and ‘Rhizaria’. A robust relationship between two main clades of the supergroup chromalveolates: Heterokonta (stramenopiles) and alveolates, with Rhizaria was recently reported (Burki et al., 2007).

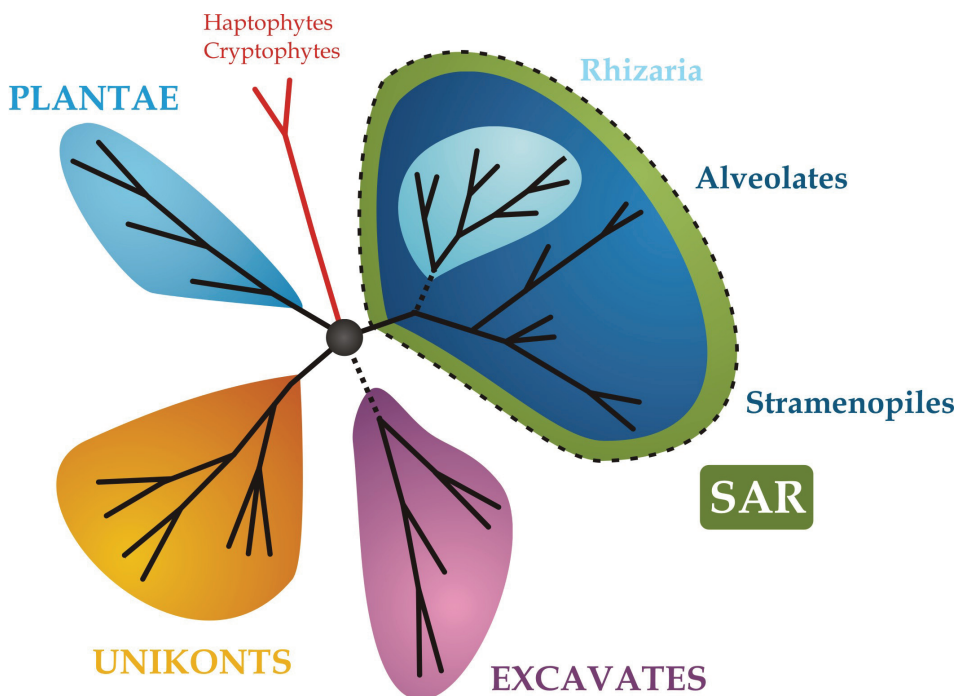


Fig. 1: Heterokonta is closely related to Alveolata and Rhizaria (Burki et al., 2007).
Illustration: Burki F. (pers. com).

Heterokonta was established as a phylum by Cavalier-Smith (1986), comprising all eukaryotic motile biflagellate cells having an anterior flagella (cilia) with tripartite rigid tubular flagellar hairs (mastigonemas) and posterior hairless (smooth) flagella, plus all

their descendants that have secondarily lost one or both flagella. Another definition uniting protozoa having evenly spaced tripartite tubular flagellar hairs under the definition Stramenopiles (Latin, *stramen*, straw, and *pilus*, hair) was given by Patterson (1989), but this definition was later regarded synonymous with Heterokonta (Cavalier Smith, 1993). Heterokonts include an amazing variety of organismal types, from the colourless flagellate *Cafeteria*, the parasite *Labyrinthulea*, and oomycetes to plastid-containing groups, including a huge variety of single-celled diatoms and giant kelps, whose thalli are parenchymatous. Due to the diversity in Heterokonta it was later raised to infrakingdom (Cavalier-Smith, 1997) with two main groups, the first being Ochrophyta (Cavalier-Smith, 1986) consisting mainly of autotrophic heterokonts. And secondly a purely heterotrophic group, which was again further subdivided in two phyla: Bigyra and Pseudofungi (Cavalier-Smith and Chao, 2006).

Classes within Heterokonta demonstrate an enormous diversity (e.g. Bacillariophyceae) and embrace several ecologically important algal (e.g. diatoms, brown algae, chrysophytes) groups. Since the erection of Heterokonta (1986) effort has been put into resolving the phylogenetic relationships among this diverse group of organisms. Analyses using different nuclear or chloroplast encoded DNA markers (Ben Ali et al., 2002; Ben Ali et al., 2001; Daugbjerg and Andersen, 1997; Edvardsen et al., 2007) were carried out in order to understand the evolutionary relationships within Heterokonta. Recently, the most species rich phylogeny of all three heterokont phyla (Ochrophyta, Bigyra, Pseudofungi) employing a comprehensive SSU rDNA dataset was performed (Cavalier-Smith and Chao, 2006). SSU rDNA sequences originating from uncultured marine heterokont flagellates have also been included in phylogenetic analyses revealing an amount of yet unidentified heterotrophic heterokont taxa (Kolodziej and Stoeck, 2007). In spite of these efforts the main branching order of Heterokonta has remained unresolved.

As a result of the SSU rDNA analysis with main emphasis on heterotrophic heterokonts, a model of possible heterokont evolution based on several morphological characters, mode of living and molecular SSU rDNA was presented by Cavalier Smith (2006) (Fig. 2).

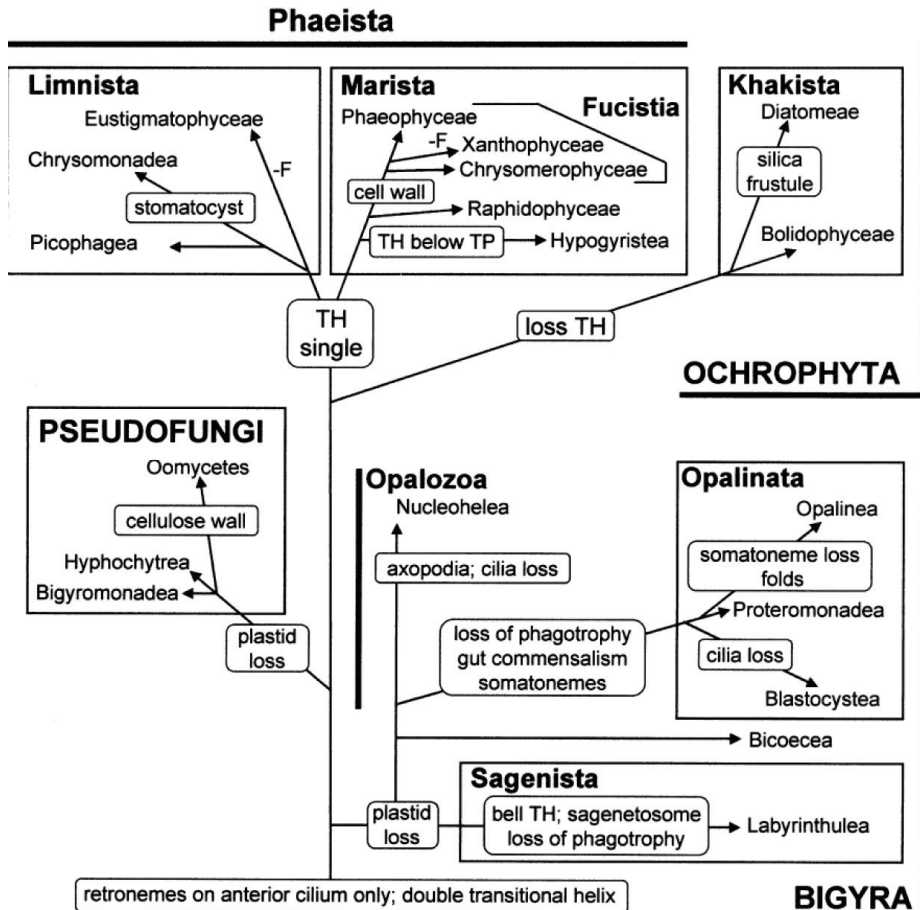


Fig. 2: Proposed model of phylogenetic relationships among heterokont classes, from Cavalier Smith (2006). The division Heterokonta is divided in three phyla (Ochrophyta, Bigyra and Pseudofungi). Bigyra is further divided into three subphyla (Sagenista, Bicoecia and Opalozoa) and Ochrophyta into two subphyla (Khakista and Phaeista). Phaeista is subdivided into infraphylum Limnista (predominantly freshwater) and Marista (predominately marine). TH = flagellar transition helix, TP = flagellar transition plate, -F = loss of fucoxanthin.

The phyla Bigyra (Cavalier-Smith, 1997) and Pseudofungi comprise heterotrophic heterokonts (Cavalier-Smith and Chao, 2006), whereas, Ochrophyta (Cavalier-Smith T., 1986) embraces mainly autotrophic heterokonts. It is important to stress that the taxonomy in Cavalier-Smith and Chao (2006) and Fig. 2 not were based solely on the new data and

analyses reported, but on integration of these data with relevant previously published data, both morphological and molecular.

1.3 Classes Raphidophyceae and Dictyochophyceae

The presence of partite tubular hairs is a characteristic of Heterokonta, but the morphology of the hairs and their distribution on the flagella differ among taxa (Cavalier-Smith and Chao, 2006; Cavalier-Smith T., 1986).

The heterokont class Raphidophyceae is characterized by having an extensive flagellar root system, sometimes including a characteristic layered structure (Vesk and Moestrup, 1987) and no distal or proximal helix in the transition region of the flagella. They may have a rhizoplast, but lack flagellar swellings (Andersen, 2004; Heywood, 1990; Heywood and Leedale, 2000). Species within the genus *Chattonella* is further characterized by a cytoplasm clearly divided into a cytoplasmatic endoplasm and a vacuolated ectoplasm, and osmiophilic granules in the peripheral cytoplasm that are visible in electron micrographs of *Chattonella* species. Mucocysts are common in many species belonging to the class Raphidophyceae, e.g. *Chattonella globosa* Y. Hara et Chihara and *Fibrocapsa japonica* (Fukuyo et al., 1990).

The heterokont class Dictyochophyceae is characterized by inconspicuous or no flagellar roots, basal bodies in a depression of the nucleus, one transitional plate and a proximal two-gyre helix (no rings) in the flagellar transition zone. They have no rhizoplast. The heterokont class Dictyochophyceae consisted until this work of three orders (Dictyochales (silicoflagellates), Pedinellales and Rhizochromulinales). The phylogenetic localization of Dictyochophyceae within Ochrophyta has not been precisely resolved, but several phylogenetic analyses have clustered Dictyochophyceae and Pelagophyceae together with high statistical support for this sister taxa affiliation (Ben Ali et al., 2002; Ben Ali et al., 2001). The class Dictyochophyceae was also classified in Hypogyrystea and has been systematically placed together with among other classes Raphidophyceae in the infraphylum Marista (Cavalier-Smith and Chao, 2006) see Fig. 2.

1.4 The genus *Pseudochattonella*

A heterokont flagellate formed in 1998 a massive bloom off the coasts of Germany, Denmark, Sweden and Norway that killed 350 tons of farmed fish (Aure *et al.*, 2001; Backe-Hansen *et al.*, 2001). The responsible organism resembled the '*Chattonella verruculosa*' described from Japan by Hara *et al.* (1994, but see also Fukuyo *et al.*, 1990). Due to distinctly unequal tripartite heterokont flagella inserted into a shallow depression near the anterior end of the cell, no visible flagellar roots as well as lack of contractile vacuoles and eyespot the reference strain of *C. verruculosa*, NIES670 (originally isolated from Seto Inland Sea in Japan) was placed in the heterokont class Raphidophyceae (Hara *et al.*, 1994). The heterokont flagellate that bloomed in Skagerrak (1998) differed somewhat from *C. verruculosa* in cell size, form and growth pattern. It was therefore tentatively named *Chattonella* aff. *verruculosa*, and was initially believed to belong to the heterokont class Raphidophyceae.

In February-March 2001 '*Chattonella* aff. *verruculosa*' bloomed again and caused the death of 1100 tons of farmed fish along the Norwegian south coast. A satellite image was taken 2001.03.25 and gives an indication of the biomass of phytoplankton in surface water during the late stage of this bloom. Along the Norwegian south coast the concentrations of phytoplankton was high, reaching up to 60 mg chlorophyll a m^{-3} (Fig. 3). Five monoalgal strains were isolated off the Norwegian south-eastern coast from this bloom and made it possible to study and characterize this phytoflagellate.

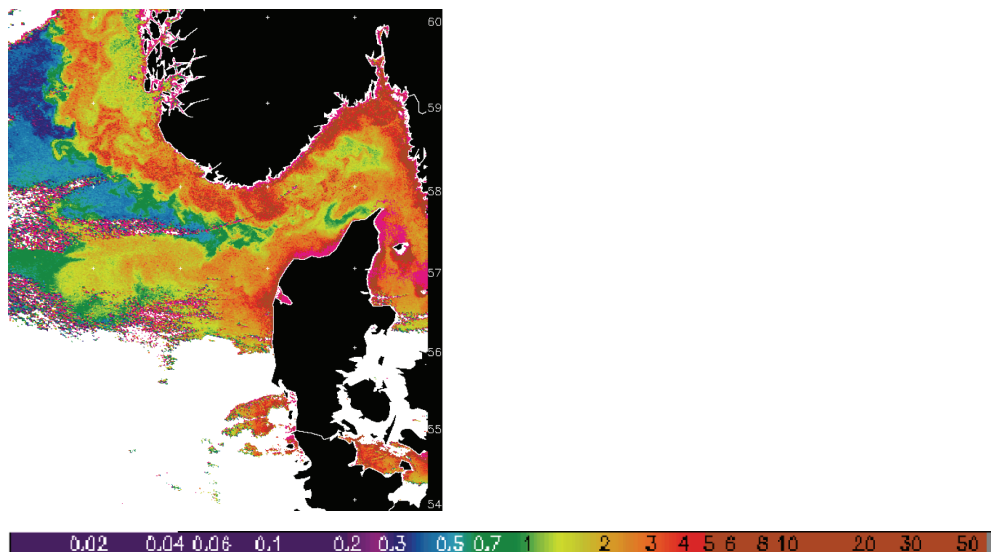


Fig. 3 Satellite image from 2001.03.25 indicate the biomass of phytoplankton (chlorophyll a mg m^{-3}) in surface water during the late stage of the bloom. White areas are cloud covered and violet areas indicate that the data were outside the range of chlorophyll algorithm. Copyright NASA SeaWiFS project Team / Orbital Imaging corp. by courtesy of remote sensing group, Plymouth Marine Laboratory, U.K and Nansen Environmental and remote sensing Centre, Norway

Blooms of '*C. aff. verruculosa*' in North Atlantic waters without fish mortalities were observed in 2000 (German Bight and off the Danish west coasts, Göbel J. & Lu, 2000), in 2002 (German Bight, off the Danish west coast and Skagen), and in 2004 (Danish coast and Kattegat, Bengt Karlsson, pers. com). To enable studies of phylogeny, geographic distribution, bloom dynamics and toxic effects in nature for *C. verruculosa* and *C. aff. verruculosa*, it was necessary to be able to separate them and gain information on the genetic variation within and between these organisms.

Since *Chattonella verruculosa* previously has been reported from Japan in 1987 (Yamaguchi et al., 1997) and 1993 (Honsoi Tanabe pers. com) as well as from New Zealand in 2003 (Rhodes Lesley, pers com.) a hypothesis of the introduction of this species with ballast water from Japan was raised (Hopkins, 2001). Ballast water as a transport vector for toxic microalga is beyond doubt (Bolch and de Salas, 2007), and has been reported for several cyst forming species within Dinophyta (Bolch and de Salas, 2007). However, phytoplankton species that do not form cysts are also capable of surviving ballast transit as the presence of viable cells of the *Aureococcus anophagefferens*

Hargraves and Sieburth (Pelagophyceae) in ships' ballast water and small-boat bilge and live-well water has been demonstrated (Doblin *et al.*, 2004).

For practical reasons, I find it necessary to here present one main result of this thesis: “The species name *Verrucophora farcimen* sp. et gen. nov. was proposed for the identified flagellate blooming in the Skagerrak (paper I). This name then had to be changed to *Pseudochattonella farcimen*”.

2 Objectives

The main objectives of this thesis were:

- To determine the phylogeny and systematic position of the genus *Pseudochattonella*.
- Genetically characterize *Pseudochattonella farcimen* and related species.
- Determine genetic diversity within and between *Pseudochattonella* species.
- Develop molecular methods for detection of *Pseudochattonella*.
- Identify the bloom-forming *Pseudochattonella* species in various geographical regions.
- Infer the global phylogeny of heterokonts.

In an effort to resolve the branching order of Heterokonta a multigene phylogenetic analysis of heterokonts using DNA (nucleotides) and protein (amino acids) sequence data was carried out (paper IV). *Pseudochattonella* was included as one of the heterokont taxa. The phylogenetic position of *Pseudochattonella* and its closest relatives were also determined from morphology in combination with rDNA sequence analysis in paper I, which is a first description of *Verrucophora farcimen*, later renamed *Pseudochattonella farcimen*. Genetic variation in three different cell compartments (chloroplast, nuclear, mitochondria) was investigated and compared between the two *Pseudochattonella* species (paper II). Further the identity of *Pseudochattonella* strains from different geographical regions were determined (paper II) and finally molecular methods for specific detection of these two species were developed and tested on environmental samples (paper III).

3 Materials and methods

3.1 Algal cultures

Five monoalgal, non-axenic strains of *Pseudochattonella* were isolated from the bloom off the south coast of Norway in March 2001. Two strains of *Pseudochattonella* were also isolated during this PhD project by single cell capillary isolation from a bloom in Skagerrak 2006. The cultures were grown in a modified half-defined medium termed IMR $\frac{1}{2}$ (Eppley et al., 1967) with salinity 25, at temperatures 4-15°C and at a photon fluence rate of 50-100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Additional 17 heterokont strains were obtained from other culture collections and kept under growth conditions recommended by their original collection (see paper IV).

3.2 Molecular markers

To identify species relationship a diverse array of molecular markers is currently available. The rate of sequence evolution varies extensively with gene or DNA segment. Finding the appropriate DNA marker for the question of interest is thus very important. One of the regions that have been extensively used in phylogenetic studies of different organisms is the nuclear ribosomal DNA cistron (Hillis and Dixon, 1991). The nuclear genes encoding the cytoplasmic ribosomal RNAs (rDNA) are in most eukaryotes organized into transcriptional rDNA units with a small (18S/SSU), a 5.8S, and a large (28S/LSU) subunit rDNA region, separated by internal transcribed spacer regions ITS1 and ITS2. The rDNA sequences are homogenized by concerted evolution, and primarily through gene conversion events among the multiple copies. The DNA sequences for LSU and SSU rDNA are under strong stabilizing selection due to their critical role in ribosome synthesis. Non-coding regions, like the ITS rDNA regions are not under similar functional constraints. As a consequence, due to faster accumulation of mutations, these regions usually show higher variability. Non-coding regions have therefore been used to study intraspecific genetic variation (Bakker et al., 1992; Connell, 2000; Lundholm et al., 2006).

Several plastid genes (e.g. *psbA*, *rbcL*) have been widely used as phylogenetic markers (Bachvaroff et al., 2005; Daugbjerg and Andersen, 1997; Wee et al., 1996). The

rubisco spacer has been used for phylogenetic studies of populations of marine algae (Andersen and Bailey, 2002; Bailey and Andersen, 1999; Rohfritsch et al., 2007; Varela-Alvarez et al., 2007).

DNA barcoding is applied to identify species of organisms by using a short (750bp) DNA sequence from a standard and agreed-upon position in the genome. The mitochondrial gene encoding the cytochrome c oxidase subunit 1 (*cox1* also referred to as COI) has emerged as the standard barcode region for higher animals (Ratnasingham and Hebert, 2007) and marine life (www.coreocean.org). Ehara et al. (1997) showed that *cox1* also has appropriate variability to resolve higher order relationships among heterokonts.

Several highly expressed protein coding genes such as *actin*, *beta-tubulin*, *elongation factors* as well as *heat-shock* proteins have been shown useful for phylogenetic inference in multigene approaches (Fast et al., 2002; Harper et al., 2005; Kim et al., 2006; Nosenko and Bhattacharya, 2007; Simpson et al., 2006). Sequence information from whole genome sequencing projects as well as other high throughput sequencing initiatives has produced an overwhelming amount of sequence data. This opens up for possibilities for larger scale multigene phylogenies where more than 70 genes can be applied (Burki and Pawlowski, 2006).

In this thesis I have applied several markers such as SSU rDNA, LSU rDNA, *actin*, *beta-tubulin*, *cox1*, heat-shock protein 90 and *rbcL*.

3.3 Ribosomal oligonucleotide probes

An oligonucleotide probe is a short sequence of nucleotides (usually 18-25 bp) synthesized to match a specific DNA region. The oligonucleotide probe hybridizes to a specific DNA region and is often coupled to a detection system, and can be useful as a tag to detect the presence of a specific DNA fragment. This principle has been used for species identification of several phytoplankton species (e.g. Lundholm et al., 2006; Not et al., 2002). In this thesis I have developed oligonucleotide probes for the specific detection of *Pseudochattonella*.

3.4 Phylogenetic analysis

A phylogenetic tree is a mathematical structure which is used to model the actual evolutionary history of a group of sequences or organisms. The task of molecular phylogeny is to convert information in sequences into an evolutionary tree. A great (and ever increasing) number of methods have been described for doing this. The most commonly used methods can be classified into three major groups; parsimony methods, likelihood methods, and distance methods. In maximum parsimony (MP) analysis, the tree(s) that requires the fewest character state changes is considered the best representation of the true phylogenetic tree (Kithching, 1998). In maximum likelihood (ML) methods, the likelihood of observing a given set of sequence data for a specific substitution model is maximized for each tree topology, and the topology that gives the highest maximum likelihood is chosen as the final tree (Nei and Kumar, 2000). MrBayes is another approach for reconstructing phylogeny and is based on Bayes' theorem which states, "Bayes' formula shows how a person who started out with one set of beliefs, formulated in the prior probability of the tree, and modifies his or her belief in the light of new data". Bayesian methods are closely related to other likelihood methods e.g. ML analysis which searches for the tree that maximizes the likelihood of the data given an evolutionary model. In distance methods, evolutionary distances are computed for all pairs of taxa, and a phylogenetic tree is constructed by considering the relationships among these distance values. Several methods for testing the reliability of each node in an inferred tree have been presented; the most commonly used is bootstrap analysis (Felsenstein, 1985). In this thesis I have applied ML, NJ, MP as well as MrBayes analyses for phylogenetic inference.

4 Results and discussion

4.1 The genus *Pseudochattonella*

In paper I we showed that *C. aff. verruculosa* from Skagerrak was genetically and morphologically different but closely related to *C. verruculosa* from Japan. The species name *Verrucophora farcimen* sp. et gen. nov was proposed for the identified flagellate blooming in Skagerrak. The genus name *Verrucophora* was, however, regarded as a later synonym of *Pseudochattonella* (Hosoi-Tanabe *et al.*, 2007), and Eikrem *et al.* (submitted) proposed a recombination of *Verrucophora farcimen* to *Pseudochattonella farcimen* Eikrem, Edvardsen and Throndsen.

Pseudochattonella was found to hold several of the features characterizing Dictyochophyceae such as inconspicuous or no microtubular roots, basal bodies in a depression of the nucleus, one transitional plate and a proximal two-gyre (two rings) helix in the flagellar transition zone, and no rhizoplast. Like in *Dictyocha*, the nucleus was located in the central to anterior part of the cell with a Golgi body alongside the anterior part of the nucleus. In addition, *Pseudochattonella* cells had a bulge on the hairy flagellum. The genus *Pseudochattonella* was from phylogenetic analyses of heterokont SSU rDNA and concatenated SSU+LSU rDNA data systematically placed within the heterokont class Dictyochophyceae (paper I, IV). Due to morphological differences such as distinct fibrous roots connecting the basal bodies and microtubular roots, and both a distal and proximal transition helix in the flagellar transition zone in *Pseudochattonella* (and *Florenciella parvula* Eikrem) a new order within the Dictyochophyceae, Florenciellales, were proposed to embrace the three species *P. farcimen*, *P. verruculosa* and *F. parvula* (Edvardsen *et al.*, 2007; Eikrem *et al.*, 2004). The two *Pseudochattonella* species (*P. verruculosa* and *P. farcimen*) were similar in ultrastructure, but in *P. farcimen* we found the nucleus branched and not rounded as in *P. verruculosa* (paper I). Further, the flagellar hairs in *P. verruculosa* were possibly bipartite, not tripartite. The presence of tripartite hairs could however, not be precluded as this structure can be difficult to reveal. The pigment composition of *P. farcimen* was investigated (paper I) and was found to be similar to other Dictyochophytes. In conclusion, ultrastructure, morphology and pigment composition supported and confirmed that the genus *Pseudochattonella* do not belong to the class Raphidophyceae, as

previously suggested (Hara et al., 1994). This conclusion is in congruence with Bowers et al.(2006) and Hosoi-Tanabe et al. (2007).

In light of paper IV, transfer of the genus *Pseudochattonella* from the class Raphidophyceae to the class Dictyochophyceae (paper I) is essential, as the two classes apparently are more distantly related than previously anticipated. Raphidophyceae was in topologies of previous SSU rDNA and concatenated SSU+LSU rDNA trees clustered together with Pelagophyceae and Dictyochophyceae, but with low statistical support for this sister taxa relationship (Ben Ali et al., 2002; Cavalier-Smith and Chao, 2006). We showed with higher support in paper IV that Raphidophyceae clustered with Phaeophyceae, Phaeothamniophyceae and Xanthophyceae whereas, Dictyochophyceae received a basal placement within Ochrophyta together with Pelagophyceae and Bacillariophyceae.

4.2 Two species of *Pseudochattonella*, unveiling patterns of genetic variability

In comparison to paper I (using nuclear rDNA) further genetic evidence for a separation of the two *Pseudochattonella* species (*P. farcimen* and *P. verruculosa*) was found in protein coding sequences from additional two cell compartments (mitochondria and chloroplast, paper II). Nuclear encoded ribosomal DNA and plastid encoded genes have for several years successfully been applied to identify species to infer phylogenetic relationships and to reveal genetic diversity (e.g. Ben Ali *et al.*, 2002; Andersen & Bailey, 2002; Ki & Han, 2007). Ribosomal DNA has the same function in all organisms and occur in high copy numbers of rDNA genes in the genome (Brown et al., 1972), further many rDNA sequences are available for sequence comparison in GenBank, and therefore rDNA is frequently chosen for studies of genetic variation (Andersen and Bailey, 2002; Ben Ali et al., 2002; Ki and Han, 2007). Due to strong stabilizing selection LSU and SSU rDNA are widely used at the species level and above. We tested the usefulness of nuclear (partial LSU rDNA, SSU rDNA and ITS rDNA) as well as mitochondrial *cox1* and chloroplast (*psbA*, *rbcL* and *rbcL-rbcS* spacer region) for the applicability of differentiating between the two species in the genus *Pseudochattonella* (paper II). We found five of the tested molecular markers (partial LSU rDNA, SSU rDNA, partial *cox1*, *psbA* and *rbcL*) useful

for investigation of intraspecific genetic variation in *Pseudochattonella* (Fig. 4). Two of these regions were further used for specific identification at the genus and species level (paper III). Well known primers for amplification of nuclear ribosomal DNA (18S and 28S) as well as the “universal” PCR primers for the *cox1* region were tested (paper I and II). In addition we designed PCR primers for amplification of *psbA* and *rbcL* regions in *Pseudochattonella* (paper II). Compared to *cox1* the three markers (*psbA*, *rbcL* and LSU rDNA) showed lower variability when sequences from *P. farcimen* were compared with those of *P. verruculosa*. The *rbcL-rbcS* spacer region was not considered useful for species delineation as it was identified as a conserved invariable marker within the genus. For identification of *Pseudochattonella* at genus level we found the SSU rDNA useful for probe development. In a microarray assay for microalgae with SSU rDNA targeted probes it was found that the region with best accessibility was in the first 1000 bases of the molecule (Medlin L. pers. com.). Four of our developed SSU rDNA probes targeted sites within the first 700 bases of the rDNA molecule and are therefore also considered useful in a future microarray detection method for multiple microalgae. For detection at species level we found the ITS1 rDNA region useful for design of species specific PCR primers (paper III).

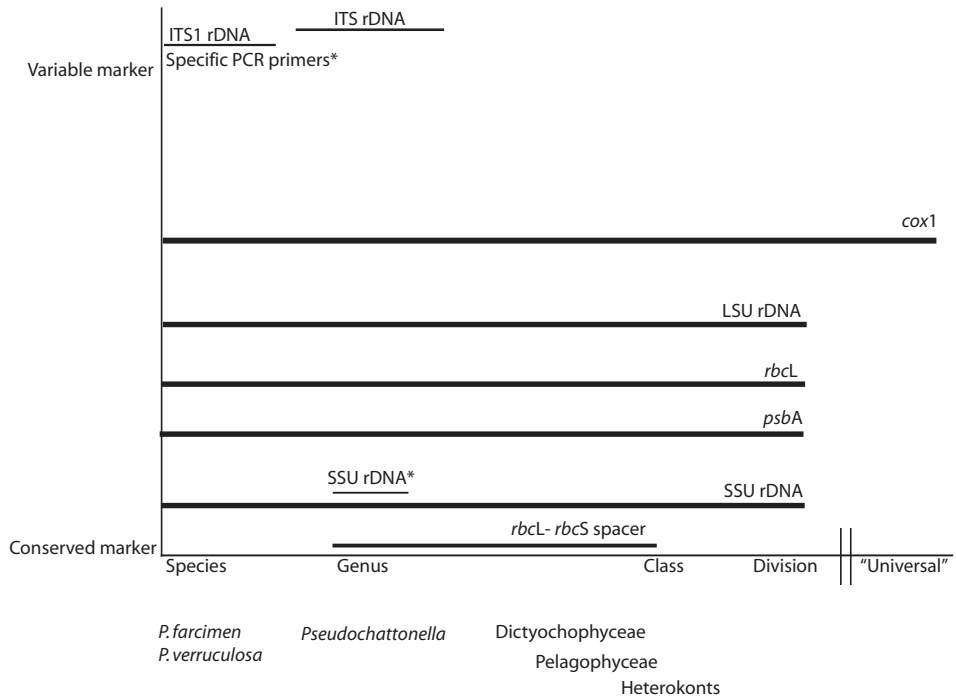


Fig. 4. Five of the tested molecular markers (partial LSU rDNA, SSU rDNA, partial *cox1*, *psbA* and *rbcL*) were useful for investigation of intraspecific genetic variation in *Pseudochattonella*. The markers *rbcL-rbcS* spacer and SSU rDNA were the most conserved markers. The variability in the remainder markers increased in the order *psbA*, *rbcL*, LSU rDNA and *cox1*. The ITS rDNA region varied within and between species of *Pseudochattonella* as well as within a single *Pseudochattonella* cell. Two of regions (SSU rDNA and ITS rDNA) were further used to develop molecular tools for specific identification of *Pseudochattonella* at the genus and species level. An asterisk (*) indicates developed PCR primers or probes specific for *Pseudochattonella* at the species of genus level.

At the level of intraspecific variation we found by sequence comparison that *P. farcimen* strains within a bloom and between blooms in successive years (2001 and 2006) were identical in the four DNA regions LSU rDNA, *cox1*, *psbA*, *rbcL* (paper II). This result suggested the presence of a homogeneous and stable population of *P. farcimen* in Skagerrak over a five years period (paper II).

Polymorphism in the SSU rDNA and ITS rDNA regions were revealed. Direct sequencing of PCR products, using either purified or diluted products, resulted in unresolved chromatograms often with multiple peaks (Fig. 5). When sequenced clones of the ITS rDNA regions in *Pseudochattonella* were compared we found the ITS1-ITS2 rDNA to vary within a species (intraspecific) both in *P. farcimen* and *P. verruculosa*. Intraclonal sequence variation was found in all *Pseudochattonella* strains examined, both in strains originating from single cell capillary isolation, and strains obtained by serial dilutions. The length of the ITS1-ITS2 rDNA region varied as well, whereas the 5.8S rDNA region was of equal length for all clones. The observed sequence variation was not correlated with geographical location or strain.

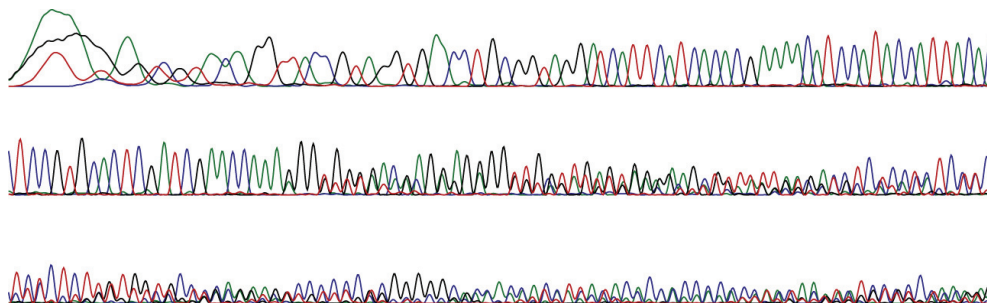


Fig. 5. Example of an unresolved chromatogram resulting from directly sequencing of PCR products from ITS1 rDNA in *Pseudochattonella farcimen*.

As part of a multigene family, the individual repeats of the nuclear ribosomal DNA arrays were expected to become rapidly homogenized through the mechanisms of concerted evolution. In our results from *Pseudochattonella*, however, multiple peaks were observed in the chromatograms of ITS rDNA (Fig. 5) and SSU rDNA, reflecting intraspecific and intraclonal variation. The sequence variation in the ITS rDNA regions of *Pseudochattonella* was markedly higher compared to that in the SSU rDNA region. We uncovered extreme high levels of ITS rDNA polymorphism in *Pseudochattonella*, this phenomena has also previously been encountered in other algal divisions (Fama et al., 2000; Rehnstam-Holm et al., 2002) as well as within heterokonts (Alverson and Kolnick, 2005). The observed intra-specific and intra-individual ITS-polymorphism arose probably due to distinct ITS rDNA haplotypes together in single *Pseudochattonella* cells. The ITS

rDNA was therefore eliminated as a marker to differentiate among individuals and/or populations of *Pseudochattonella*.

Concerted evolution is generally accepted as a universal phenomenon (Liao, 1999). In recent years, however, scientists have begun to realize the extent of both intraspecific and intra-individual variability in nuclear ribosomal genomes (Gribble and Anderson, 2007). Eukaryotic genomes contain in general more than one copy of the rDNA genes. The rDNA copy number has from quantitative PCR experiments been shown to vary among phytoplankton species and be highly correlated with cell length (Zhu et al., 2005). From Fig. 6, where heterokont species is highlighted in red colour we see variation in copy numbers of rDNA among heterokont species from below 10 copy numbers in *Nannochloropsis salina* Hibberd (Eustigmatophyceae) and *Pelagomonas calceolans* (Pelagophyceae) to copy numbers in the order of 40-1000 in *Thalassiosira* (Bacillariophyceae). From our results of heterokont phylogeny (paper IV), observation of ITS rDNA polymorphism in *Pseudochattonella* (paper II) combined with the information presented in (Gribble and Anderson, 2007) and the reported variation in rDNA copy number between heterokont groups (Zhu et al., 2005), I believe heterokonts in general, and especially *Pseudochattonella*, could be potential candidates for further studies of molecular mechanisms of the homogenization effects arising from concerted evolution.

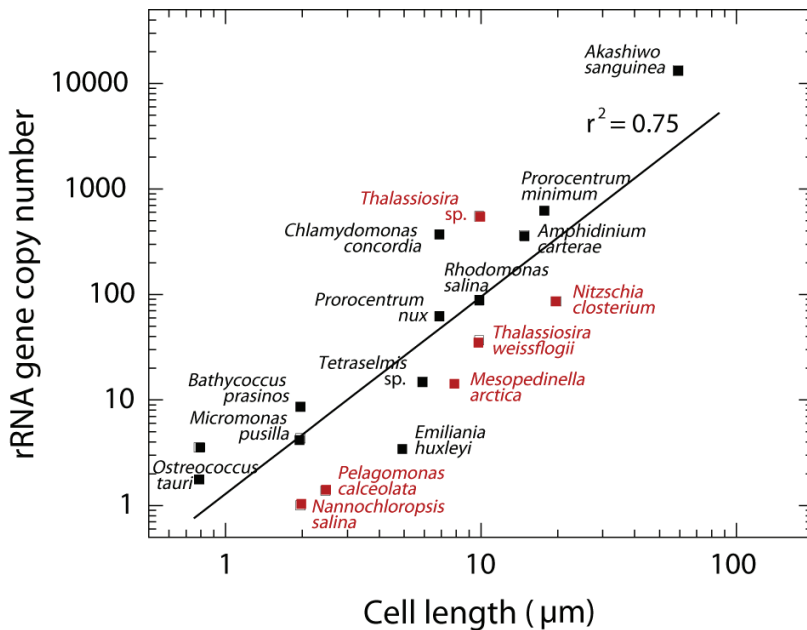


Fig. 6. Correlation between rDNA copy number estimated by quantitative PCR and cell length from 18 strains of phytoplankton modified from (Zhu et al., 2005). Heterokont species are emphasized in red color.

4.3 Biogeographic distribution of

Pseudochattonella

For studies of biogeographic distribution, bloom dynamics, and toxic effects of *Pseudochattonella* in nature it is necessary to be able to separate the two *Pseudochattonella* species. In this aspect information on the variation within a species is valuable knowledge. We have developed species specific PCR primers and oligonucleotide probes for DotBlot hybridization (paper III) and shown these molecular tools to be useful for detection of *Pseudochattonella* to the species level. *Pseudochattonella verruculosa* were identified in Germany 2000, New Zealand 2003 (paper II) as well as in environmental samples from Netherlands 2006 (paper III) whereas, *P. farcimen* were identified in the Skagerrak in 2006 and 2007 samples (paper II, III). This was the first record of *P. farcimen* in the autumn season, since all previous records were from winter-spring (January-May). We have shown (paper III) that the probes and specific primers are valuable tools in studies of geographical and seasonal distribution of *Pseudochattonella* to the species level.

Real-time PCR is a promising molecular approach for detection and quantification purposes (Bowers et al., 2006; Coyne et al., 2005). If it is desirable to distinguish between the two species of *Pseudochattonella* without sequencing I suggest a single nucleotide polymorphism (SNP) approach for species differentiation. This can be carried out through a real-time PCR approach (Roche 480 Basic software, Mannheim, Germany v.1.2). The principle of SNP analysis in a Roche 480 LightCycler is based on detection of differences in the melting points by fluorescence of hybridization probes. A perfectly matching hybridization probe will melt at a higher temperature than a probe with one mismatch bound to the target sequence. From the sequence information available from this thesis it is possible to design a hybridization probe of *P. verruculosa* that has one base pair mismatch compared to the *P. farcimen* DNA sequence in combination with specific PCR primers for *Pseudochattonella*. The melting point of the *P. verruculosa* probe will then have a slightly lower melting point compared to a perfectly matched probe of *P. farcimen*. This principle has been widely used for genotyping (Saito et al., 2007) and I think it could be useful for species differentiation of *Pseudochattonella*.

While the role of ballast water as a transport vector for toxic microalgae is now beyond doubt (Bolch and de Salas, 2007), finding definitive proof of the introduction of any organism, particularly microorganisms, is exceedingly difficult. Through this project we have used molecular tools to gain knowledge about *Pseudochattonella farcimen* causing blooms in Skagerrak, and we have also determined the identity of *Pseudochattonella* from other geographical locations. From the limited numbers of *Pseudochattonella* strains available it was a difficult scope of this thesis to approach the question whether *P. farcimen* is an introduced species or not (paper II). With the tools now available, also for detection of *Pseudochattonella* directly in environmental samples (paper III), this opens up for future studies addressing the question of introduction.

4.4 Heterokont phylogeny

Multigene approaches for resolving phylogenetic relationships using a moderate number of protein encoding genes have during the last few years been successfully applied to several protist groups (Fast et al., 2002; Kim et al., 2006; Nosenko and Bhattacharya, 2007; Simpson et al., 2006). For heterokonts, two gene analyses (SSU+LSU rDNA) have been performed - but only for the heterokont phyla Ochrophyta and Pseudofungi, with main emphasis on Ochrophyta (Ben Ali et al., 2002; Edvardsen et al., 2007). In paper IV we have therefore performed a two gene SSU+LSU rDNA analysis (3906 nucleotides) of all three heterokont phyla (Ochrophyta, Pseudofungi and Bigyra). Further we added protein sequences from four genes (*actin*, *β -tubulin*, *cox1* and *hsp90*) to the SSU+LSU rDNA dataset, and thereby generated the most gene rich heterokont alignment to date.

In all our inferred global heterokont trees (paper IV) the split between heterotrophic heterokonts (Bigyra and Pseudofungi) and Ochrophyta was strongly supported in contrast to earlier phylogenies (Cavalier-Smith and Chao, 2006). Despite the amount of data (5126 characters) concatenating six genes in a multigene analysis we were not able to resolve the phylogeny of heterotrophic heterokonts. In a seven gene (the *rbcL* gene added) phylogenetic analysis of Ochrophyta all main branches of Ochrophyta were resolved, except the position of Chrysophyceae, Eustigmatophyceae, and Pinguiphyceae.

Our trees embracing the heterokont phyla Ochrophyta, Bigyra and Pseudofungi indicated that the heterotrophic heterokonts (Bigyra and Pseudofungi) have a higher rate of evolution (inferred from branch length) than Ochrophyta. Among all tested models of

sequence evolution the covarion model (Huelsenbeck, 2002) fitted the data with the highest negative log likelihood. Application of the covarion model on the multigene data resulted in a tree with better support for the basal branches of heterokonts than earlier shown in studies of global heterokont phylogeny (Ben Ali et al., 2002; Cavalier-Smith and Chao, 2006; Edvardsen et al., 2007).

From our molecular data in combination with current knowledge about ultrastructure of heterokonts, we suggest a possible scenario of heterokont evolution (Fig. 7) that differs somewhat from previous views (Fig. 2 (Cavalier-Smith and Chao, 2006)). Our results gave reason to move Dictyochophyceae and Raphidophyceae from subphylum Phaeista to Khakista. From all our trees, in combination with current knowledge about ultrastructure of heterokonts we suggest that a more advanced flagellar apparatus originated at one occasion in the ancestor of Phaeista whereas, Khakista independently reduced their flagellar apparatus and gained chlorophyll C₃. Two heterotrophic lineages are depicted (Fig. 7). We believe at least two heterotrophic heterokont clades exist. Since the number of heterokont taxa was relatively limited (paper IV), it is likely that more data from other groups are needed to further resolve the heterokont tree.

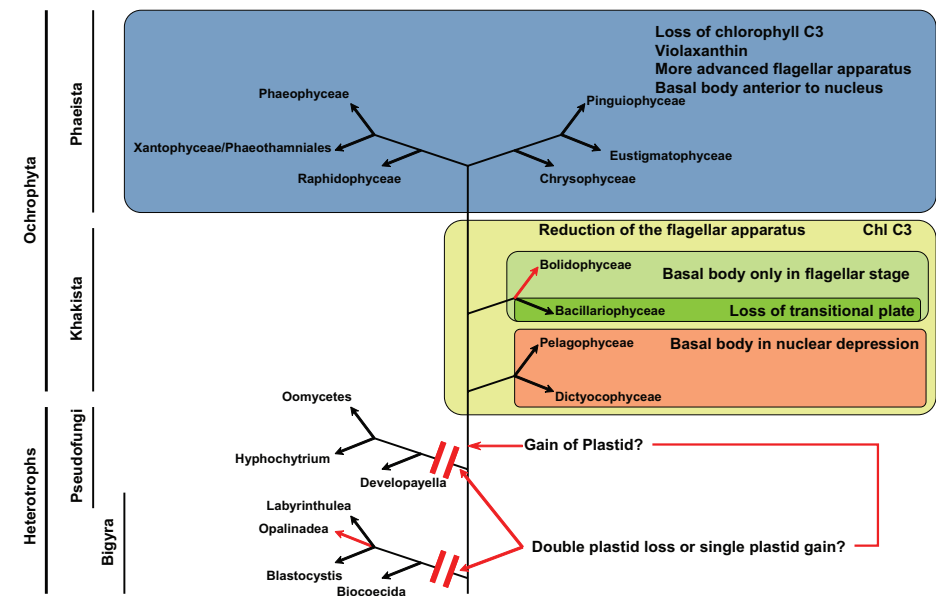


Fig. 7 Suggested phylogenetic relationships between classes of heterokonts analyzed in this study

As the majority of information in our combined rDNA and protein alignments were on the rDNA data, we believe it is worth generating an ever larger alignment with more genes and increased number of heterotrophic taxa, also from yet unexplored heterotrophic heterokonts (Kolodziej and Stoeck, 2007). Extending our analyses with improved taxon and gene sampling – combined with ultrastructural characters - will enable us to better understand the evolution of this diverse group of organisms.

5 Future perspectives

5.1 Expressed sequence tag library of *P.*

farcimen

According to numbers given at the NCBI website (<http://www.ncbi.nlm.nih.gov>) of April 2006, there were over 130 billion bases in GenBank and RefSeq alone. Although the genomic information in general is increasing there has been a lack of genomic information available for the heterokont class Dictyochophyceae. Until date, molecular data available from Dictyochophytes were 11 *rbcL* protein sequences and 69 nucleotide sequences (ribosomal DNA (rDNA) and *rbcL*) in NCBI databases (March 2008).

In collaboration with Alfred Wegner Institute for Polar and Marine Research, an Expressed Sequence Tag (EST) dataset of *Pseudochattonella farcimen* (strain UIO 109) was obtained through this PhD project. The EST dataset has been assembled with the software phred phrap (Ewing and Green, 1998) and functionally annotated by Blast2GO (<http://www.blast2go.de/>). Some of the EST sequences (of *P. farcimen*) were included in the multigene phylogenetic analysis of heterokonts as a representative of the class Dictyochophyceae (paper IV). Over 10.000 EST clones were sequenced, and of these 2390 were assembled into so called contigs (continuous sequences) by phred phrap (Table 1). ESTs that cannot be assembled by other ESTs are called singeltons.

Table 1: Distribution of EST sequences into contigs and singeltons (phred phrap assembly)

	No. of sequences
Total number of ESTs	10367
Singeltons (EST not part of a contig)	3174
	out of which 2355 was of acceptable length
ESTs in tentative unique contigs	7193
TCs (Tentative unique sequences)	2390

5.2 Functional annotation

Gene Ontologies (GO) gives information of how gene products behave in a cellular context. GO annotations are divided into three main categories, biological process, cellular component and molecular function. Out of 4745 sequences (2390 contigs and 2355 singeltons) 1057 sequences were annotated and assigned a function by Blast2GO. These sequences were further mapped to a subset of GO terms into broader categories. Initial studies of annotated EST sequences of *P. farcimen* revealed that genes responsible for catabolic processes as well as protein metabolic processes and reproduction were abundant in the annotated EST sequences. The EST library gives future possibilities of insight into transcriptionally active regions in the genome of *P. farcimen*. The percentage of unknown genes in the *P. farcimen* EST dataset was 55% when compared to the NCBI database and 61% when compared to the SwissProt database (November 2007). With respect to the novelty rate of the ESTs, *P. farcimen* is until now the first transcriptome dataset available for the class Dictyochophyceae.

Pelagophyceae and Dictyochophyceae are closely related classes within the heterokonts. This has previously through several phylogenetic analyses been suggested with high statistical support (Ben Ali et al., 2002; Cavalier-Smith and Chao, 2006) and is also supported by similarities in ultrastructure. The relationship of these groups as sister taxa has also been confirmed through multigene analysis in this thesis (paper IV). The genome of *Aureococcus anophagefferens* (Pelagophyceae) is currently in the process of sequencing at DOE Joint Genome Institute (<http://www.jgi.doe.gov/>), making a more comprehensive comparison of these two datasets realistic in near future. As an increasing number of sequence information of heterokonts become available, such as EST libraries and whole genome projects (e.g. oomycetes (*Phytophthora infestans*), Bacillariophyceae (*Thalassiosira pseudonana*), Pelagophyceae (*Aureococcus anophagefferens*), Phaeophyceae (*Laminaria digitata*), Eustigmatophyceae (*Nannochloropsis oculata*) and Labyrinthulea (*Schizochytrium* sp.) this opens up for future possibilities to compare sequence information from several heterokont classes and find unique genes for the class Dictyochophyceae. The EST library generated for *Pseudochattonella farcimen* will in this respect be important as to represent the class Dictyochophyceae. The 4745 tentative unique sequences will also give valuable insight into transcriptionally active regions in *P. farcimen*.

References

- Alverson AJ, and Kolnick L** (2005) Intragenomic nucleotide polymorphism among small subunit (18S) rDNA paralogs in the diatom genus *Skeletonema* (Bacillariophyta). *J. Phycol.* **41**: 1248-1257
- Andersen RA** (2004) Biology and systematics of heterokont and haptophyte algae. *Am. J. Bot.* **91**: 1508-1522
- Andersen RA, and Bailey JC** (2002) Phylogenetic analysis of 32 strains of *Vaucheria* (Xanthophyceae) using the *rbcL* gene and its two flanking spacer regions. *J. Phycol.* **38**: 583-592
- Bachvaroff TR, Puerta MVS, and Delwiche CF** (2005) Chlorophyll c-containing plastid relationships based on analyses of a multigene data set with all four chromalveolate lineages. *Mol. Biol. Evol.* **22**: 1772-1782
- Bailey JC, and Andersen RA** (1999) Analysis of clonal cultures of the brown tide algae *Aureococcus* and *Aureoumbra* (Pelagophyceae) using 18S rRNA, *rbcL*, and rubisco spacer sequences. *J. Phycol.* **35**: 570-574
- Bakker FT, Olsen JL, Stam WT, and Vandenhoek C** (1992) Nuclear Ribosomal DNA Internal Transcribed Spacer Regions (ITS1 and ITS2) define discrete biogeographic groups in *Cladophora albida* (Chlorophyta). *J. Phycol.* **28**: 839-845
- Behrenfeld MJ, and Falkowski PG** (1997) Photosynthetic rates derived from satellite-based chlorophyll concentration. *Limnol. Oceanogr.* **42**: 1-20
- Ben Ali A, De Baere R, De Wachter R, and Van de Peer Y** (2002) Evolutionary relationships among heterokont algae (the autotrophic stramenopiles) based on combined analyses of small and large subunit ribosomal RNA. *Protist* **153**: 123-132
- Ben Ali A, De Baere R, Van der Auwera G, De Wachter R, and Van de Peer Y** (2001) Phylogenetic relationships among algae based on complete large-subunit rRNA sequences. *Int. J. Syst. Evol. Microbiol.* **51**: 737-749
- Bolch CJS, and de Salas MF** (2007) A review of the molecular evidence for ballast water introduction of the toxic dinoflagellates *Gymnodinium catenatum* and the *Alexandrium "tamarensis" complex* to Australasia. *Harmful Algae* **6**: 465-485

- Bowers HA, Tomas C, Tengs T, Kempton JW, Lewitus AJ, and Oldach DW** (2006) Raphidophyceae [Chadefaud ex Silva] systematics and rapid identification: sequence analyses and real-time PCR assays. *J. Phycol.* **42** 1333-1348
- Brodiet J, and Lewis J** (2007) Unravelling the algae the past, present, and future of algal systematics. The Systematics Association special volume series 75 CRC press
- Brown DD, Wensink PC, and Jordan E** (1972) Comparison of ribosomal DNAs of *Xenopus laevis* and *Xenopus mulleri*: Evolution of tandem genes. *J. Mol. Biol.* **63**: 57-73
- Burki F, and Pawlowski J** (2006) Monophyly of Rhizaria and multigene phylogeny of unicellular bikonts. *Mol. Biol. Evol.* **23**: 1922-1930
- Burki F, Shalchian-Tabrizi K, Minge MA, Skjæveland Å, Nikolaev SI, Jakobsen KS, and J. P** (2007) Phylogenomics reshuffles the eukaryotic supergroups. *PlosOne* **8**:790, 1-6
- Cavalier-Smith T** (1986) The kingdom Chromista: origin and systematics. Progress in phycological research **Biopress, Bristol**: 309-347
- Cavalier-Smith T** (1997) Sagenista and Bigyra, two phyla of heterotrophic heterokont chromists. *Archiv Für Protistenkunde* **148**: 253-267
- Cavalier-Smith T and Chao EEE** (2006) Phylogeny and megasystematics of phagotrophic heterokonts (kingdom Chromista). *J. Mol. Evol.* **62**: 388-420
- Cavalier-Smith T.** (1986) The kingdom Chromista: origin and systematics. Progress in phycological research **Biopress, Bristol**: 309-347
- Cavalier Smith T** (1993) Kingdom Protozoa and its 18 Phyla. *Microbiological Reviews* **57**: 953-994
- Connell LB** (2000) Nuclear ITS region of the alga *Heterosigma akashiwo* (Chromophyta : Raphidophyceae) is identical in isolates from Atlantic and Pacific basins. *Mar. Biol.* **136**: 953-960
- Coyne KJ, Handy SM, Demir E, Whereat EB, Hutchins DA, Portune KJ, Doblin MA, and Cary SC** (2005) Improved quantitative real-time PCR assays for enumeration of harmful algal species in field samples using an exogenous DNA reference standard. *Limnology and Oceanography-Methods* **3**: 381-391
- Daugbjerg N, and Andersen RA** (1997) A molecular phylogeny of the heterokont algae based on analyses of chloroplast-encoded *rbcL* sequence data. *J. Phycol.* **33**: 1031-1041

- Edwardsen B, Eikrem W, Shalchian-Tabrizi K, Riisberg I, Johnsen G, Naustvoll L, and Throndsen J** (2007) *Verrucophora farcimen* gen. et sp. nov (Dictyochophyceae, Heterokonta) - A bloom-forming ichthyotoxic flagellate from the Skagerrak, Norway. *J. Phycol.* **43**: 1054-1070
- Ehara M, Hayashi-Ishimaru Y, Inagaki Y, and Ohama T** (1997) Use of a deviant mitochondrial genetic code in yellow-green algae as a landmark for segregating members within the phylum. *J. Mol. Evol.* **45**: 119-124
- Eikrem W, Romari K, Larasa M, Le Gall F, Throndsen J, and Vaultot D** (2004) *Florenciella parvula* gen. et sp. nov (Dictyochophyceae, Heterokontophyta), a small flagellate isolated from the English Channel. *Phycologia* **43**: 658-668
- Eppley RW, Holmes RW, and Paasche E** (1967) Periodicity in cell division and physiological behaviour of *Ditylum brightwellii*, a marine planktonic diatom during growth in light-dark cycles. *Arch. Mikrobiol.*, **56**: 305-323
- Ewing B, and Green P** (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res.* **8**: 186-194
- Fama P, Olsen JL, Stam WT, and Procaccini G** (2000) High levels of intra- and inter-individual polymorphism in the rDNA ITS1 of *Caulerpa racemosa* (Chlorophyta). *Eur. J. Phycol.* **35**: 349-356
- Fast NM, Xue LR, Bingham S, and Keeling PJ** (2002) Re-examining alveolate evolution using multiple protein molecular phylogenies. *J. Eukaryot. Microbiol.* **49**: 30-37
- Felsenstein J** (1985) Confidence-limits on phylogenies - an approach using the bootstrap. *evolution* **39**: 783-791
- Fukuyo Y, Takano H, Chihara M, and Matsuoka K** (1990) Red tide organisms in Japan: An illustrated taxonomic guide. **Uchida Rokakuho, Tokyo, Japan** 407pp
- Gribble KE, and Anderson DM** (2007) High intraindividual, intraspecific, and interspecific variability in large-subunit ribosomal DNA in the heterotrophic dinoflagellates *Protoperidinium*, *Diplopsalis*, and *Preperidinium* (Dinophyceae). *Phycologia* **46**: 315-324
- Hara Y, Doi K, and Chihara M** (1994) Four new species of *Chattonella* (Raphidophyceae, Chromophyta) from Japan. *J. Phycol.* **42**: 407-420
- Harper JT, Waanders E, and Keeling PJ** (2005) On the monophyly of chromalveolates using a six-protein phylogeny of eukaryotes. *Int. J. Syst. Evol. Microbiol.* **55**: 487-496

- Henriksen P** (1993) Autecology, life history and toxicology of silicoflagellate *Dictyocha speculum* (Silicoflagellate, Dictyochophyceae). *Phycologia* **32** 29-39
- Heywood P** (1990) Phylum Raphidophyta. In Margulis, L., Corliss, J.O., Melkonian, M. and Chapman, D. J. [Eds.] *Handbook of Protoctista*. Jones and Bartlett, Boston 318–25
- Heywood P, and Leedale GF** (2000) Order Raphidomonadida Heywood & Leedale, 1983. In Lee, J. J., Leedale, G. F. and Bradbury, P. [Eds.] *The Illustrated Guide to the Protozoa*. 2nd ed. Vol. 2. Society of Protozoologists, Lawrence, Kansas 744-751
- Hillis DM, and Dixon MT** (1991) Ribosomal DNA - molecular evolution and phylogenetic inference. *Q. Rev. Biol.* **66**: 411-453
- Hopkins CCE** (2001) A review of introductions and transfers of alien marine species in the North Sea area. Report to Directorate for Nature Management (DN), DN Oslo Norway, Available at:
<http://www.dirnat.no/archive/attachments/01/04/CHMDA071.pdf>.
- Hosoi-Tanabe S, Honda D, Fukaya S, Otake I, Inagaki Y, and Sako Y** (2007) Proposal of *Pseudochattonella verruculosa* gen. nov., comb. nov (Dictyochophyceae) for a former raphidophycean alga *Chattonella verruculosa*, based on 18S rDNA phylogeny and ultrastructural characteristics. *Phycol. Res.* **55**: 185-192
- Huelsenbeck JP** (2002) Testing a covariotide model of DNA substitution. *Mol. Biol. Evol.* **19**: 698-707
- Ki JS, and Han MS** (2007) Nuclear rDNA and chloroplast *rbcL*, *rbcS* and IGS sequence data, and their implications from the Japanese, Korean, and North American harmful algae, *Heterosigma akashiwo* (Raphidophyceae). *Environ. Res.* **103**: 299-304
- Kim E, Simpson AGB, and Graham LE** (2006) Evolutionary relationships of apusomonads inferred from taxon-rich analyses of 6 nuclear encoded genes. *Mol. Biol. Evol.* **23**: 2455-2466
- Kithching IJ** (1998) *Cladistics* 2nd Edition. Oxford University Press, Oxford, UK.
- Kolodziej K, and Stoeck T** (2007) Cellular identification of a novel uncultured marine stramenopile (MAST-12 clade) small-subunit rRNA gene sequence from a Norwegian estuary by use of fluorescence in situ hybridization-scanning electron microscopy. *Appl. Environ. Microbiol.* **73**: 2718-2726
- Liao DQ** (1999) Concerted evolution: Molecular mechanism and biological implications. *Am. J. Hum. Genet.* **64**: 24-30

- Lundholm N, Moestrup O, Kotaki Y, Hoef-Emden K, Scholin C, and Miller P** (2006) Inter- and intraspecific variation of the *Pseudo-nitzschia delicatissima* complex (Bacillariophyceae) illustrated by rRNA probes, morphological data and phylogenetic analyses. *J. Phycol.* **42**: 464-481
- Nei M, and Kumar S** (2000) *Molecular Evolution and Phylogenetics* **Oxford University Press, New York**:
- Nosenko T, and Bhattacharya D** (2007) Horizontal gene transfer in chromalveolates. *BMC Evol. Biol.* **7**: 173
- Not F, Simon N, Biegala IC, and Vaultot D** (2002) Application of fluorescent in situ hybridization coupled with tyramide signal amplification (FISH-TSA) to assess eukaryotic picoplankton composition. *Aquat. Microb. Ecol.* **28**: 157-166
- Patterson DJ** (1989) Stramenopiles: chromophytes from a protistean perspective. In: Green J. C., Leadbeater B. S. C., and Diver W. L. (eds). *The chromophyte algae: problems and perspectives*. Clarendon Press Oxford 357-379
- Ratnasingham S, and Hebert PDN** (2007) BOLD: The Barcode of Life Data System (www.barcodinglife.org). *Mol. Ecol. Notes* **7**: 355-364
- Rehnstam-Holm AS, Godhe A, and Anderson DM** (2002) Molecular studies of Dinophysis (Dinophyceae) species from Sweden and North America. *Phycologia* **41**: 348-357
- Rohfritsch A, Payri C, Stiger V, and Bonhomme F** (2007) Molecular and morphological relationships between two closely related species, *Turbinaria ornata* and *T. conoides* (Sargassaceae, Phaeophyceae). *Biochem. Syst. Ecol.* **35**: 91-98
- Saito Y, Bag SS, Kusakabe Y, Nagai C, Matsumoto K, Mizuno E, Kodate S, Suzuka I, and Saito I** (2007) Dual-labeled oligonucleotide probe for sensing adenosine via FRET: A novel alternative to SNPs genotyping. *Chemical Communications* 2133-2135
- Simpson AGB, Inagaki Y, and Roger AJ** (2006) Comprehensive multigene phylogenies of excavate protists reveal the evolutionary positions of "primitive" eukaryotes. *Mol. Biol. Evol.* **23**: 615-625
- Varela-Alvarez E, Stengel DB, and Guiry MD** (2007) Seasonal growth and phenotypic variation in *Porphyra linearis* (Rhodophyta) populations on the west coast of Ireland. *J. Phycol.* **43**: 90-100
- Vesk M, and Moestrup O** (1987) The flagellar root system in *Heterosigma akashiwo* (Raphidophyceae). *Protoplasma* **137**: 15-28

- Wee JL, Hincley JM, Nguyen KX, Kores P, and Hurley DL** (1996) Investigating the comparative biology of the heterokonts with nucleic acids. *J. Eukaryot. Microbiol.* **43**: 106-112
- Yamaguchi M, Itakura S, Nagasaki K, Matsuyama Y, Uchida T, and Imai I** (1997) Effects of temperature and salinity on the growth of the red tide flagellates *Heterocapsa circularisquama* (Dinophyceae) and *Chattonella verruculosa* (Raphidophyceae). *J. Plankton Res.* **19**: 1167-1174
- Zhu F, Massana R, Not F, Marie D, and Vaulot D** (2005) Mapping of picoeucaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. *FEMS Microbiol. Ecol.* **52**: 79-92